AN IMPROVED METHOD OF TISSUE DIGESTION FOR SPICULE MOUNTS IN SPONGE TAXONOMY

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Digestion of bioeroding sponges is difficult as the tissue of many samples cannot be dissolved easily using traditional techniques involving 70% nitric acid. A number of factors reduce the effectiveness of the acid, such as dilution by water from fresh and alcohol preserved specimens, and buffering effects, particularly contamination with calcium carbonate. In these preparations spicules are often obscured by a white precipitate and tissue/spongin residues. This traditional method was emended to produce clean preparations, with the added benefit that digestion time was greatly reduced. In contrast to repeated treatments with 70% nitric acid in an 80°C sandbath under the traditional method, I propose the use of alternate applications of 70% aqua regia and 70% nitric acid in a 140°C sandbath with whirl shaking after each application. Drying the tissue samples prior to digestion was important to speed up the process. Nevertheless, under the modified method some impurities remained resistant to acid digestion, including diatoms, clay and quartz particles. ❐ *Porifera, Demospongiae, bioeroding sponges, tissue digestion, taxonomy, spicule preparations.*

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Spicule morphology remains a fundamental criterion in sponge identification, yet their preparation for examination under light microscopy has changed very little since last century. Traditionally, demosponge spicule preparations have been obtained by digesting sponge tissue and calcareous particles in either sodium hypochlorite ('bleach') or heating in nitric acid, leaving the siliceous spicules remaining. Nitric acid digestions have been applied in several ways: 1) a small piece of tissue placed directly onto a microscope slide and digested by dropping small amounts of acid and boiling off the supernatant, with the spicules fixed in place with a mounting medium; 2) tissue digested in a test tube, then spread over slides by burning a drop of the resuspended mixture, and subsequentially fixed onto the slide with a standard mountant.

The first method, henceforth referred to as the traditional method, has been widely used over time and is adequate for sponges containing few or no foreign particles. It also has the advantage over the second method in minimising the potential loss of rare spicules or microscleres from slides, given that preparation occurs directly on the slide medium, whereas using separate platforms for digestion and viewing introduces the possibility that some spicules may be lost during their transfer to glass slides (usually via pipette). However, this traditional method is clearly inadequate for arenaceous species and bioeroding species (i.e. those that bore into calcitic substrata). Under the traditional method calcitic debris is retained on slides, obscures spicules, and often make spicule preparations too thick to be useful. The second method, henceforth referred to as the modified method, was described most recently by Schönberg & Barthel (1997, 1998), and enables clean spicules to be pipetted from the test tube onto the slide, leaving behind the contaminating material.

However, recent tests on bioeroding sponges boiled in 70% nitric acid (Schönberg, unpublished data), found both methods were unsatisfactory for this group of sponges. The present study aimed to identify the reasons for the reduced effectiveness of acid during digestion, and to develop improvements in the modified method.

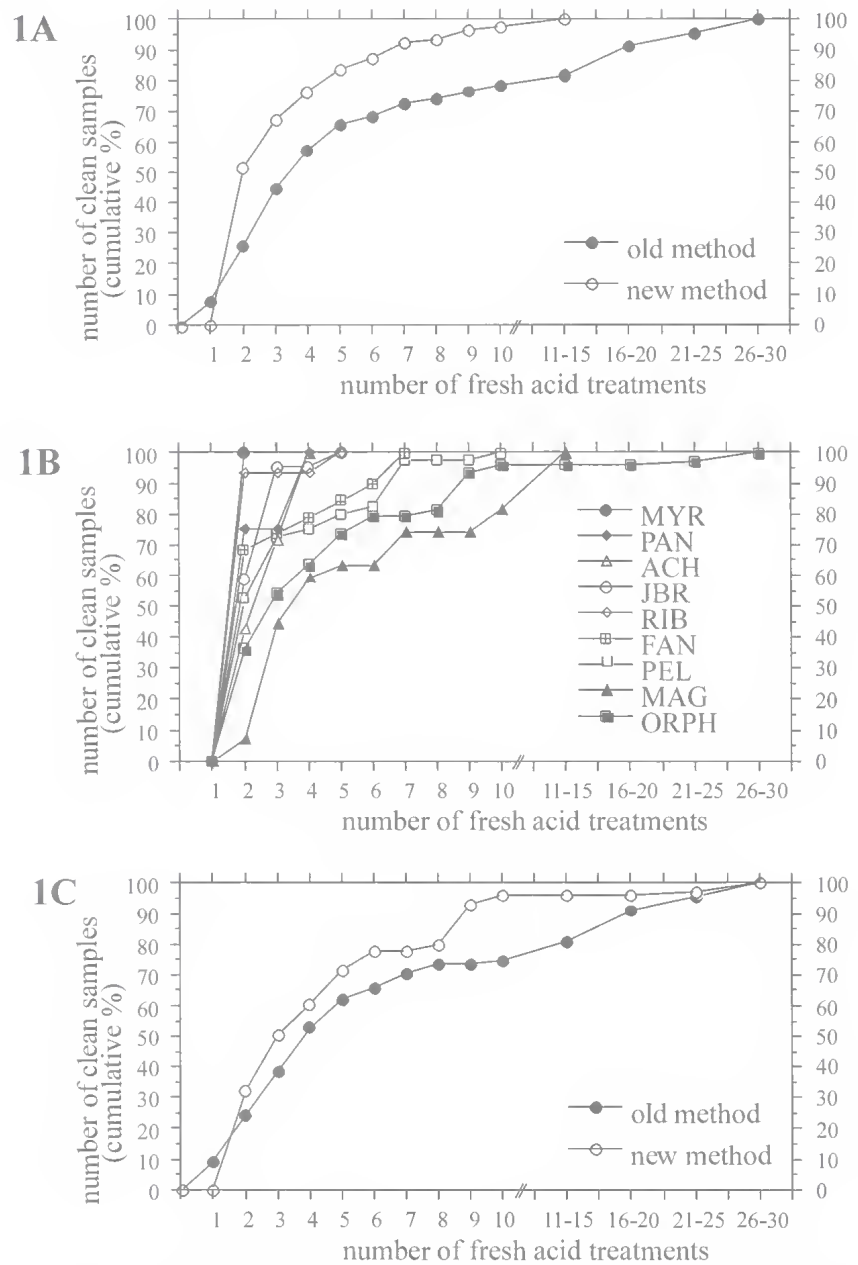

FIG. 1 A-C. Bioeroding sponge tissue digestion speed expressed in number of acid washes necessary to obtain clean spicule samples. A, Digestion speed for samples from all sample sites combined. Black circles - traditional method 2a (N=255); white circles - new method 2b (N=217); B, Digestion speed ordered by sample site (digestion under method 2b only). Black circles - Myrmidon Reef (MYR; N=14), white circles - John Brewer Reef (JBR, N=22), white rhombus - Rib Reef (RIB, N=15), white squares - Pelorus Island (PEL; N=40), black squares - Orpheus Island (ORPH; N=107), subdivided white squares - Fantome Island (FAN; N=19), white triangles - Acheron Island (ACH; N=7), black triangles - Magnetic Island (MAG; N=27), black rhombus - Pandora Reef (PAN; N=8). C, Digestion speed for samples from a single sample site, Little Pioneer Bay, Orpheus Island. Black circles - method 2a (N=200), white circles - method 2b (N=99). Values represent counts, hence no error bars are included.

TABLE 1. Relationship of bioeroding sponge tissue digestion speed and distance to the shore through possible uptake of fine terrestrial sediments.

Sample site	Distance to shore (km)	Visibility during dive (m)	% of samples clean after a given number of acid washes		
			50%	80%	100%
Myrmidon Reef	112	> 25	2	2	2
John Brewer Reef	72	~ 20	2	3	5
Rib Reef	55	~ 20	2	2	5
Fantome Island	20	5-7	2	5	7
Acheron Island	18	~ 10	3	4	4
Orpheus Island	16	< 5	3	8	>30
Pandora Reef	15	~ 5	2	2	4
Pelorus Island	14	5-7	2	5	10
Magnetic Island	5	2-5	4	10	10-15

MATERIALS AND METHODS

Bioeroding sponges were collected from the central region of the Great Barrier Reef, Queensland. Material was acid digested using three methods.

1) Traditional method of placing a small fragment of sponge in 70% nitric acid directly on a microscope slide, and boiling under low heat (e.g. alcohol flame or a sand bath). This simple traditional method is described by Hooper (1996).

2a) Modified method of Schönberg & Barthel (1997) whereby pieces of sponge tissue 1-3mm^3 were digested in test tubes using repeated washes with 70% nitric acid in an 80°C sand bath. Each application of fresh acid was allowed to react over night. This method is similar to that described by Hooper (1996) for digesting sponge tissue for examination under scanning electron microscopy.

2b) Pieces of sponge tissue 1-3mm^3 were dried at room temperature for 2 days or overnight at 80°C, then pre-digested in test tubes in a 140°C sand bath using aqua regia (1 volumetric unit of 70% nitric acid and 3 units of 70% hydrochloric acid). The high temperature was chosen to maximise acid reactivity by keeping it at boiling level (hydrochloric acid: 110°C; nitric acid: 122°C; Falbe & Regitz, 1992), allowing for lower temperatures inside the test tubes than at the bottom of the sandbath. Spent acid was removed after 24hrs and replaced by 70% nitric acid. The samples were then again left overnight in the 140°C sandbath. This combined acid treatment was repeated as required. Samples were mixed on a whirl shaker with each fresh application of acid (Fig. 1A).

Using both methods, digestion was considered to be finished when the solution looked clear (i.e. without any yellow colouration or white debris remaining in the spicule sediment). In a few cases, digestion was stopped after more than 20 acid applications even though the sample still appeared whitish, and there was no evidence of nitrous gases indicating that the acid was still reacting with organic material. The supernatant acid was removed, and residual acid in the test tubes was allowed to react off with 70% ethanol added dropwise. The re-settled spicules were washed twice with 100% ethanol to remove remaining acid and to dehydrate the sample. Spicules were stored in 100% ethanol until mounting. The proportion of sample concentrations of spicules to alcohol was adjusted to a ratio of about 1:10 in volume, or less, to ensure optimal spicule concentrations on the microscope slides when applying the same amount of spicule suspension (i.e. 300µl). The suspension was haphazardly spread on a level slide and then burnt off (Schönberg & Barthel, 1998). Before fixing the cover slip with DPX mountant, a flame was held underneath the slide to remove vapour still adhering to the spicules and the microscope slide.

The efficiency of both methods was compared by noting the number of acid washes necessary to obtain clear suspensions. In addition, the quality of the preparations was checked by noting whether microscleres or spines on megascleres were still obscured, and if so by what. In 69 sponges, ectosome and choanosome regions were sampled separately to assess whether it was possible that different proportions of spongin and cell material were responsible for producing different digestion results.

RESULTS

The traditional method of tissue digestion, as well as the modified method of Schönberg & Barthel (1997, 1998), were both found to be inadequate in producing clear, useful slide preparations for bioeroding sponge spicules, irrespective of whether digestion was attempted

directly on a microscope slide (method 1) or in a test tube (method 2a).

The traditional method (1) usually produced a thick, white precipitate on the microscope slide, which obscured the spicules, especially microscleres. It was almost impossible to obtain clean preparations, even when carefully washing with alcohol. Moreover, washing increased the risk of losing spicules.

The modified digestion method (2a) was extremely slow, requiring many fresh acid washes, and not always dissolving all the tissue. As a consequence, microscleres and spines on megascleres were often obscured, minimising their usefulness of these slides for sponge identification. With up to 20-30 acid washes there was also the risk of losing small and rare spicules during repeated pipetting off the supernatant spent acid. Using method 2b proposed here, the efficiency of sponge tissue digestion was markedly improved.

A comparison of methods 2a and 2b is as follows.

1) After two acid applications, one each of aqua regia and 70% nitric acid, spicules were clean in 50% of samples using method 2b. By comparison, using 70% nitric acid in an 80°C sand bath, an average of four acid applications were required to produce 50% of clean samples under method 2a.

2) After four acid applications 75% of samples were clean under method 2b; whereas nine applications were required to produce the same result under method 2a. After nine acid applications 95% of samples were clean under method 2b.

3) After 10 acid applications using method 2b all samples were generally clean (Fig. 2B), whereas using method 2a samples still

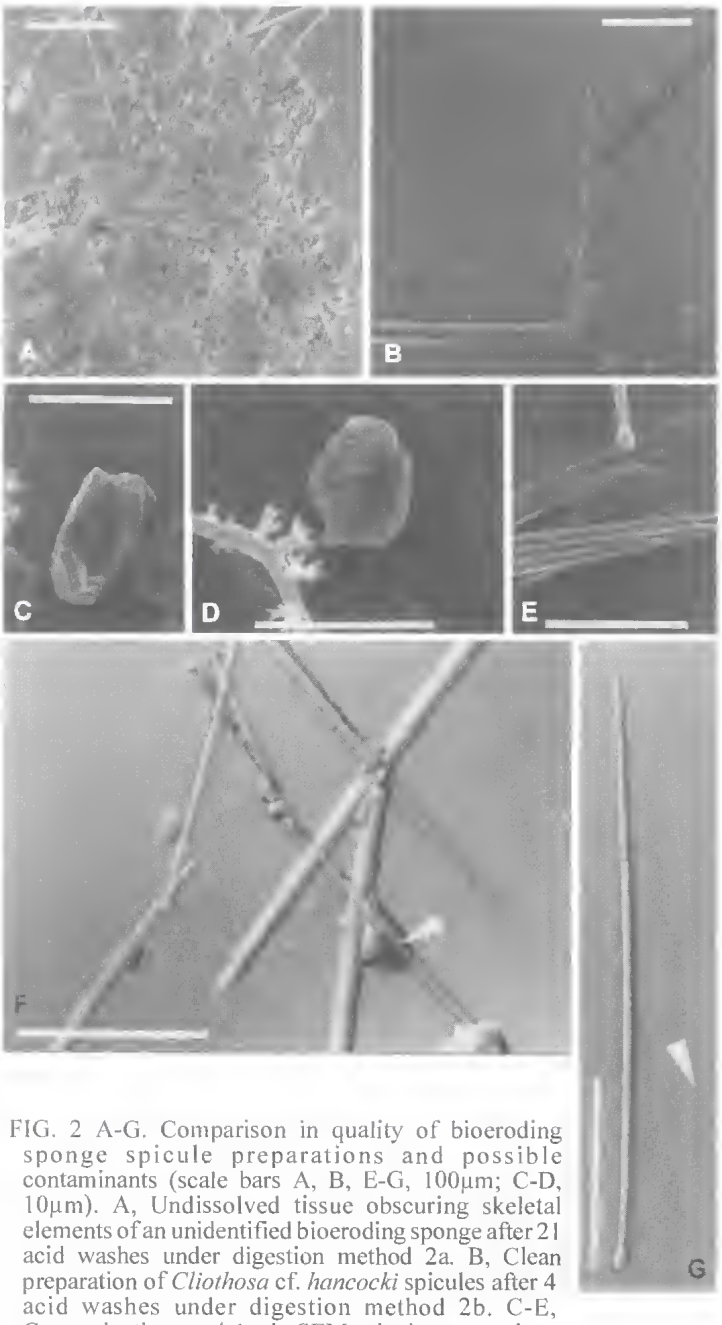

FIG. 2 A-G. Comparison in quality of bioeroding sponge spicule preparations and possible contaminants (scale bars A, B, E-G, 100μm; C-D, 10μm). A, Undissolved tissue obscuring skeletal elements of an unidentified bioeroding sponge after 21 acid washes under digestion method 2a. B, Clean preparation of *Cliothosa* cf. *hancocki* spicules after 4 acid washes under digestion method 2b. C-E, Contaminating particles in SEM spicule preparations of *Cliona viridis* sensu Bergman (1983). C, Diatom. D, Clay particle. E, Quartz particle. F, Permanent preparation of *C. viridis* skeletal elements with residual water adhering to the spicules, obscuring finer details and microscleres (arrow). G, Clear spicule preparation of *C. viridis* which has been dried by heating with a flame prior to mounting (arrow = clearly visible spirasters).

contained debris, obscuring microscleres in particular, which adhered to debris or remained embedded in tissue (Fig. 2A).

4) Under method 2a 50/255 (20%) of samples contained incompletely digested material after more than 10 acid washes, whereas only 5/217 (2%) of samples contained 'impurities' under method 2b.

Clearly, using the modified method 2b there are far fewer 'impurities' in samples than under method 2a. Moreover, any 'impurities' that did remain in spicule suspensions were inorganic debris rather than organic tissue remains. This remaining debris was checked under the electron microscope and identified as diatoms, clay and quartz particles (Ross Freeman pers. comm.; Rothwell, 1989; Fig. 2C-E). Therefore, it is possible that part of the cloudiness remaining in samples may be a product of the habitats from which samples were collected, such as differing levels of turbidity and sedimentation rates in different localities. Empirical support for this hypothesis comes from a comparison between samples collected from offshore and mid-shelf sites, which were digested much faster than samples collected from inshore sites. These latter samples were the only ones which could not be cleaned entirely of debris (Fig. 1B). This finding largely correlates with visibility recordings made during field collections, although samples from Pandora Reef and Acheron Island digested surprisingly better than expected (Table 1).

The distribution of data comparing the different methods was not random in terms of sample sites. To test for bias in site-effect, data were re-evaluated for one site (Little Pioneer Bay, Orpheus Island), which had both the lowest visibility and highest number of samples. These data provided weaker support for method 2b over 2a, than did analysis of the entire data set, although both data sets followed the same trend (Fig. 1C). For the site-effect data, methods 2a and 2b showed 50% of the samples were clear of debris after 4 and 3 acid washes, respectively, 75% after 10-15 and 6 washes, 90% after 15-20 and 9 washes, and 100% of samples were clear after more than 30 washes of acid. Whereas under method 2a 51/200 samples (26%) were still cloudy after 10 washes, in method 2a only 4/99 samples (4%) were cloudy.

Too few data were available to statistically evaluate the effect of collection localities (zones) on coral reefs, but visually the data suggest that acid digestion was slightly better from samples collected from the fore-reef zones than from lagoons or back reefs, possibly related to differential sedimentation rates between the various zones. Potential bias due to seasonal effects were not tested given the patchy collection schedule.

Differences in the type of sponge tissue did not appear to effect the speed of digestion except in *Aka* cf. *mucosa*, in which tissue samples from the soft choanosome digested on average twice as fast as those taken from of the long, brittle papillae. In general, it was found that tissue samples with minimum calcium carbonate digested better than more calcitic samples, although this is often difficult to achieve for most biocroding sponges which often incorporate calcitic debris into the choanosome. Nevertheless, some samples that contained more than 50% of their volume with incorporated coral skeleton were sometimes clean within the first two acid washes.

No differences were observed in acid digestion rates between the several different (but still unidentified) species of *Cliona* and *Aka* sampled, apart from the example of *A.* cf. *mucosa* mentioned above. In general, however, samples which contained more soft tissue (such as those eroding large chambers), generally digested slightly better than those in which tissue penetrated carbonate substrates in honeycomb patterns.

Finally, in the modified method 2b, repeated alcohol washes were necessary after digestion to avoid residual acid that masked spicule features (such as spines). These washes also reduced the amount of water in samples, as did heating microscope slides before adding the mountant, which can obscure finer details of spicules (Fig. 2F-G).

DISCUSSION

A general problem in sponge tissue digestion is the high water content of fresh tissue samples, which dilutes the acid. Similarly, the readily available 'concentrated nitric acid' is actually 70% concentrated, whereas the more effective 'fuming nitric acid' is both extremely expensive and unavailable in certain countries. However, dehydration is easily achieved by fixing samples in ethanol, or drying fresh samples, prior to acid digestion, with the latter method being the most effective.

Another explanation for the reduced effect of acid in tissue digestion is the buffering effect of

calcium carbonate debris, which is a particular problem in bioeroding sponges. Most samples examined in this study contained a relatively high proportion of calcium carbonates, particularly those that produce small excavations in which the tissue cannot be separated entirely from the host coral skeleton. In species which excavate large chambers, where tissue can be more easily be separated from carbonate, eroded coral fragments (termed "sponge chips" by Cobb (1969) are always present in the tissue.

Inorganic contaminants also occur in samples, mostly skeletal remains of diatoms, clay and quartz particles (Fig. 2C-E). Clay minerals may also produce a decreased effectiveness in acid digestion, because they are phyllosilicates, which are negatively charged and thus attract cations resulting in a specific ion-exchange capacity depending on the nature of the mineral (Brownlow, 1979). In acidic solution, these cations can be substituted against hydrogen ions until an equilibrium is reached, which could produce an additional buffering effect (Schröder, 1978).

Silicate contaminants produce a cloudy supernatant in acid digestions, superficially appearing to be a problem involving undissolved organic matter and suggesting that digestions should be repeated. This is incorrect, however, and repeated digestions with nitric acid or aqua regia do nothing further to siliceous particles given that these are chemically similar to the demosponge spicules. One solution to this problem is foresight in the design of the sampling program, armed with the knowledge that specimens living close to the shore are likely to contain higher amounts of such debris than those sampled further away (Fig. 1B, Table 1).

Tissue of bioeroding sponges was surprisingly resistant to acid digestion. There is evidence that bioeroding sponges are able to shift the calcium carbonate solubility equilibrium with the aid of carbonic anhydrase in favour of substrate dissolution ($CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow 2H^+ + CO_3^{2-}$; Pomponi, 1980). The production of hydronium ions in close vicinity to the etching cells of the sponge would require a special resistance to their corrosiveness. However, the etching process is not yet entirely explained.

The improved method for sponge tissue digestion described here is primarily based on increasing the efficiency of acid by reducing its dilution and buffering effects as far as possible.

1) The greatest improvements were gained by drying tissue samples and increasing the sand bath temperature from 80°C to 140°C.

2) Alternating rinses using aqua regia and nitric acid provided some improvement, although the reason for this is not clear. Due to the development of activated chlorine and nitrose chloride ($HNO_3 + 3\,HCl \Leftrightarrow NOCl + Cl_2 + 2H_2O$), aqua regia is more corrosive than both nitric acid or hydrochloric acid alone (Falbe & Regitz, 1990). Curiously, aqua regia alone seemed to be less efficient than when used in alternation with nitric acid. The reason for this is also not clear.

3) Repeated stirring during acid digestion, using a whirl shaker, makes some small difference to the efficiency of the process by providing a better saturation of organic shreds with acid, and by physically breaking down larger particles.

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